Isolation and functional analysis of novel secreted proteins in *Magnaporthe oryzae*

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Abstract

Rice blast disease, caused by fungus *Magnaporthe oryzae*, is one of the most serious diseases of rice. To elucidate the molecular basis of interactions between rice and *M. oryzae* upon infection, we employed a multifaceted approach that combined Robust-Long SAGE (RL-SAGE) and Massively Parallel Signature Sequencing (MPSS) sequencing method and bioinformatics analysis to identify secreted protein genes in *M. oryzae*-infected rice leaves. A total of 217 putative secreted protein genes of *M. oryzae* were found to be expressed *in planta*. Three primary candidates of secreted protein genes; MG14, MG20, and MG23 were amplified and cloned. Transient expression assay in rice protoplasts and agro-infiltration assay have been performed for functional analysis. We found that MG14 and MG20 induced cell death in rice protoplasts. Moreover, a full-length MG20 significantly induced cell death in rice protoplasts when compared with a truncated version (without signal peptide). Interestingly, agro-infiltration of a full-length MG20, but not truncated version induced cell death in *Nicotiana benthamiana*, suggesting that this protein might be active in apoplast. Protein domain search indicated that MG14 is predicted to be an extracellular membrane-associated protein with a CFEM domain. Also, a zinc binding site was found in MG20. Characterization of these fungal secreted proteins and their targets might provide new insights into the functional interaction of secreted proteins with host proteins.

Background

- ***Magnaporthe oryzae* is the causal agent of rice blast, the most devastating disease of rice worldwide.**
- **The rice/*M. oryzae* pathosystem has become a model for the study of plant-microbe interaction as both genome sequences are available[1,2].**
- **Unlike the molecular events during early stages of infection, there have very limited studies on molecular events occurring after appressoria penetration[3].**
- **Many secreted proteins of fungal pathogens delivered into their host plants are putative effector molecules which generally contribute to pathogenicity by manipulating biochemical, physiological, and morphological processes in host plants[4]. However, little is known for the function of effector proteins and the delivery mechanisms in *M. oryzae*[5].**
- **In this study, we aim to identify and characterize the secreted protein genes in *M. oryzae*-infected rice leaves by employing an integrated genomics approaches in order to elucidate the molecular basis of interactions between rice and *M. oryzae*.**

Results

Identification of *M. oryzae* secreted protein genes expressing during *M. oryzae*–rice interaction using Robust-Long SAGE (RL-SAGE) and Massively Parallel Signature Sequencing (MPSS) method

- **4,923 annotated *M. oryzae* genes were identified by the RL-SAGE and MPSS methods.**
- **A total of 217 putative secreted protein genes of *M. oryzae* were found to expressed *in planta*.**

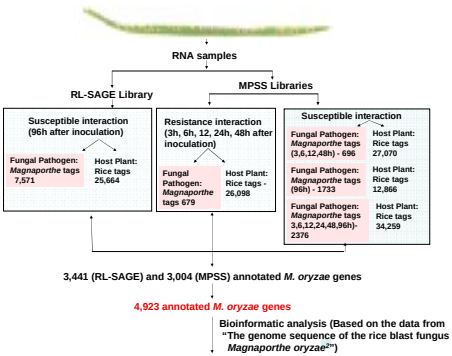


Fig. 1 Identification of *in-planta* expressed genes encoding secreted proteins of *M. oryzae* : A RL-SAGE library with RNA isolated from the rice leaf tissues after 96-h compatible blast infection and 11 MPSS libraries from both incompatible and compatible interactions were constructed. The RL-SAGE and MPSS analyses identified in total 4,923 annotated *M. oryzae* genes. Following the data from "The genome sequence of the rice blast fungus *Magnaporthe oryzae*[2]", 217 putative secreted protein genes of *M. oryzae* were found to be expressed *in planta*.

Reference

[1]International Rice Genome Sequencing Project. (2005) *Nature* **436**:793 – 800
[2]Dean, R.A., et al. (2005) *Nature* **434**:980-986
[3]Gilbert, M.J., et al. (2006) *Nature* **440**: 535-539
[4]Rep, M. (2005) *FEMS Microbiology Letters* **253**:19–27
[5]Caracuel-Rios, Z., & Talbot, N.J. (2007) *Curr Opin Microbiol* **10**:339-345
[6]Chen, S., et al. (2006) *Mol Plant Path* **7**:417–427
[7]Lee, S., et al. (2006) *MPMI* **19**:1368-1377

Cloning of *M. oryzae* secreted protein genes *in-planta*

- **31 *in planta* expressed secreted protein genes were cloned using a versatile, TA cloning technique combined vector system.**

+ **PCR amplification from ESTs, or RNA samples from rice leaves after infection.**

Fig. 2 A vector system based on a TA cloning technique. By digestion with the *Ahd* I and removing the small fragment (the gray color). The linear vector with single 3'T overhang is ideal for cloning PCR fragments with single 3'A overhang

Transient expression assay of *M. oryzae* secreted protein genes in rice protoplasts

- **3 primary candidates of secreted protein genes; MG14, MG20, and MG23 were cloned and were transiently expressed in rice protoplasts by following Chen, S. et al. protocol[6].**
- **MG14, MG20 significantly induced cell death in rice protoplasts.**

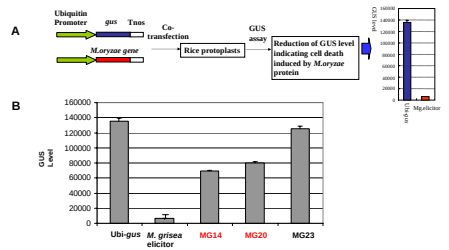


Fig. 3 Transient expression assay of *M. oryzae* secreted protein encoding genes in rice protoplasts: (A) Rice protoplasts were co-transfected with Ubi-*gus* and *M. oryzae* gene constructs for transient expression assay. Reduction of GUS level indicating cell death induced by *M. oryzae* protein; (B) Overexpression of 2 *M. oryzae* genes (MG14, MG20) induced cell death in rice protoplasts.

Transient expression assay of *M. oryzae* secreted protein genes in *Nicotiana benthamiana*

- **A full-length and truncated version (without signal peptide) constructs of 3 primary candidates of *M. oryzae* secreted protein encoding genes (MG14, MG20, and MG23) were agro-infiltrated in *N. benthamiana*.**
- **Agro-infiltration of a full-length MG20, but not truncated version induced cell death in *N. benthamiana*, suggesting that this protein might be active in apoplast.**

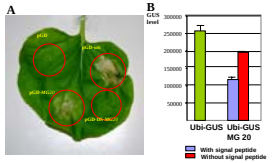


Fig. 4 The full length of *M. oryzae* secreted protein gene MG20 induced cell death in both *Nicotiana benthamiana* and rice protoplasts.

(A) A full length and truncated version (without signal peptide) of MG20 were cloned in pGD vectors, then agro-infiltrated in *N. benthamiana*. Empty pGD vector was used as negative control, and pGD containing *wts* gene was used as positive control.
(B) A full length and truncated version (without signal peptide) of MG20 were transient expressed in rice protoplasts. A full length but not truncated version of MG20 induced cell death in rice protoplasts and correlated with the result of transient expression of this gene in *N. benthamiana*.

Yeast secretion trap for characterizing secreted proteins

- **By using a yeast secretion trap system modified from Lee et al.[7], the secretion of *M. oryzae* secreted proteins can be confirmed.**

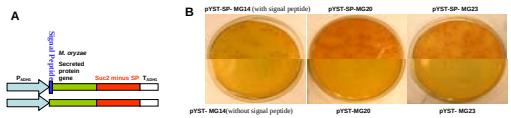
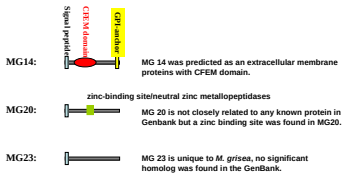


Fig. 5 Modified yeast secretion trap (pYST) cDNA library vectors and their use to identify fungal secreted proteins. (A) Schematic diagram of the YST vectors pYST-0, used for cDNA library construction. The positions of the promoter (PADH1) and terminator (TADH1) of the alcohol dehydrogenase gene are indicated. *suc2* represents a yeast invertase gene lacking its own signal peptide (SP) and initiator methionine.
(B) YST analysis of *M. oryzae* MG14, MG20, and MG23; the yeast transformed with fused *M. oryzae* genes with predicted signal peptides grew on sucrose medium, indicating secretion function of the *M. oryzae* genes.

Blast and Domain/Motif search of 3 identified *M. oryzae* proteins



Conclusions

- **The RL-SAGE and MPSS analysis identified 4,923 annotated *M. oryzae* genes from rice blast-infected rice leaf tissue. Among them, 217 *in-planta* expressed putative secreted protein genes of *M. oryzae* were identified.**
- **By employing the highly efficient cloning vector, 31 *in-planta* expressed *M. oryzae* secreted protein genes were cloned. Three primary candidates of secreted protein genes; MG14, MG20, and MG23 were tested in rice cells by using rice protoplast transient expression system. The cell-death inducing putative effector protein genes were identified.**
- **Transient expression of *M. oryzae* secreted protein genes by agro-infiltration assay in *N. benthamiana* suggests that the secreted protein gene MG20 might be active in apoplast.**
- **Prediction of *M. oryzae* secreted proteins can be confirmed by the yeast secretion trap system.**

Future direction

Functional studies of the *M. oryzae* secreted proteins:

- **Identification of interacting host targets of the *M. oryzae* secreted proteins**
- **Overexpression/knockdown of effector genes in *M. oryzae* and target genes in rice**

Acknowledgements

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